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Autocrine activation of the IFN signaling pathway may promote immune escape in glioblastoma

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Abstract

Background: Interferons (IFN) are cytokines typically induced upon viral infection, but are constitutively expressed also in the absence of acute infection. The physiological role of autocrine and paracrine IFN signaling however remains poorly understood, and its function in glioblastoma has not been explored in depth.

Methods: Using RNA interference-mediated gene silencing we characterized constitutive type I IFN signaling and its role in human glioma cells.

Results: We observed constitutive expression of pSTAT1 and MxA, a classical IFN-response marker, in the absence of exogenous IFN-β. *In vivo*, we found higher MxA expression in gliomas than in normal tissue suggesting that IFN signaling is constitutively active in these tumors. To demonstrate the presence of an autocrine type I IFN signaling loop in glioma cells *in vitro*, we first confirmed the expression of the type I receptor, IFNAR1/2, and its ligands, IFN-α and IFN-β. siRNA-mediated receptor gene silencing resulted in reduced expression of MxA at mRNA and protein level, as did gene silencing of the ligands, corroborating the hypothesis of an autocrine signaling loop in which type I IFN induce intracellular signaling through IFNAR1/2. On a functional level, following *IFNAR1* or *IFNAR2* gene silencing, we observed reduced PD-L1 and MHC class I and II expression as well as an enhanced susceptibility to NK immune cell lysis, suggesting that autocrine IFN signaling contributes to the immune evasion of glioma cells.

Conclusions: Our findings point to an important role of constitutive IFN signaling in glioma cells by modulating their interaction with the microenvironment.

Key words: glioma, interferon, immunogenicity, PD-L1, STAT1

Importance of the study:

Interferons (IFN) might be constitutively expressed also in the absence of acute infection thereby regulating various physiological processes such as cellular proliferation and differentiation, cell viability as well as immune cell function. However, their role in glioblastoma, especially in glioma-initiating cells, has not been investigated in detail. Here we show that IFN signaling is constitutively active in gliomas *in vitro* and *in vivo*. We demonstrate the presence of an autocrine signaling loop in which the type I IFN, IFN-α and IFN-β, induce intracellular signaling through the type I IFN receptor, IFNAR1/2. On a functional level, we define the potential impact of constitutive IFN signaling on the immunogenicity of glioma cells. *IFNAR1/2* gene silencing reduces PD-L1 and MHC class I and II expression and enhances susceptibility to immune cell lysis, suggesting that constitutive IFN signaling acts as a negative regulator of anti-tumor immune responses in gliomas.

Introduction

Gliomas are intrinsic brain tumors which represent a major clinical challenge. Despite intense therapeutic efforts, these tumors typically progress and ultimately result in neurological deterioration and death. This unfavorable prognosis reflects the biological properties of glioma cells which are paradigmatic for various hallmarks of cancer such as invasive growth, impaired immunogenicity and resistance to various apoptotic stimuli [1,2]. The underlying mechanisms are only partially understood but is has become clear that various alterations on the genetic and molecular level contribute to the malignant behavior of glioma cells. Furthermore, the existence of a subpopulation of cells that harbor stem-cell characteristics within gliomas suggests that a rather small percentage of the tumor cells may maintain tumor growth [3]. With regard to stem cells, type I interferons (IFN) such as IFN-α or IFN-β have gained increasing interest within the last years. Constitutive type I IFN signaling may be important for the maintenance and mobilization of hematopoietic stem cells within the niche since either the absence of constitutive IFN signaling or prolonged elevated IFN signaling deplete the hematopoietic stem cell niche. Upon chronic IFN signaling, an induction of proliferation was observed in dormant hematopoietic stem cells, a finding that might be of relevance also for cancer stem cells [4,5].

IFN are produced by most nucleated cells and their signaling is mediated through a common cell surface type I IFN receptor complex composed of two subunits, IFNAR1 and IFNAR2 [6,7]. IFNAR2 is supposed to be responsible for ligand binding, while IFNAR1 holds very weak ligand binding affinity, but induces intracellular signaling cascades [8-10]. The ligand-mediated association of the two subunits promotes a signaling cascade that results in the phosphorylation of IFNAR1 and creation of a docking site for STAT2. STAT2 phosphorylation creates a docking site for STAT1 which enables phosphorylation of STAT1 [7]. The STATs either form homodimers of STAT1, or heterodimers of STAT1 and STAT2

which then translocate to the nucleus to induce expression of IFN-stimulated genes. Additionally, type I IFN can activate other members of the STAT family such as STAT3, STAT4, STAT5 or STAT6 [11].

Activity of the IFN-signaling pathway also leads to the induction of myxovirus A (MxA) protein expression, a cytoplasmic GTPase with antiviral activity [12]. The genes activated by IFN do not only play a crucial role in cellular processes protecting from viral infections, but also in modulating general immune responses, cell proliferation and cell survival [13]. Moreover, IFN signaling might be of relevance also in different tumor types since mutations, preventing the production of or altering the responsiveness to IFN, have been observed in numerous malignancies like leukemia or melanoma, thus possibly representing a survival advantage for tumor cells [14,15]. In addition, the efficacy of type I IFN as an anti-cancer treatment is affected by constitutive IFN expression [16]. Thus, based on the involvement of type I IFN in various cellular processes, we aimed at characterizing constitutive IFN signaling and its role in glioma cells including glioma cells with stem cell properties.

Materials and methods

Cell lines and reagents

The human long-term cell (LTC) lines U87MG and T98G were obtained from the American Type Culture Collection (Manassas, VA), and LN-18, LN-428, D247MG, LN-319, A172, LN-308 and LN-229 cells were kindly provided by N. de Tribolet (Lausanne, Switzerland). The glioma-initiating cells (GIC) T-269, T-325, S-24, ZH-161 and ZH-305 were isolated from tumors resected in Tübingen, Germany (T-269, T-325), Stuttgart, Germany (S-24), or Zurich, Switzerland (ZH-161, ZH-305) and were used during the first passages [17]. Cells were authenticated regularly at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany by Short Tandem Repeat (STR) analysis, lastly in 2013 (LTC) and in 2016 (GIC). All LTC were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS, Invitrogen) and 2 mM glutamine. The GIC cultures were maintained in Neurobasal (NB) medium (Invitrogen) supplemented with B-27 supplement (20 µl/ml), Glutamax (10 µl/ml) (Invitrogen), fibroblast growth factor (FGF)-2, epidermal growth factor (EGF) (20 ng/ml each; Peprotech, Rocky Hill, PA) and heparin (32 IE/ml; Ratiopharm, Ulm, Germany). The natural killer cell line NKL was obtained from M.J. Robertson (Indiana University School of Medicine, Indianapolis, IN) and cultured in RPMI 1640 medium (Invitrogen) supplemented with 15% FCS, 2 mM glutamine and 50 U/ml interleukin (IL)-2 (Peprotech). Recombinant IFN-β1b was purchased from AbD Serotec (Düsseldorf, Germany). Antibodies to human CD45 (clone 2B11) was from DakoCytomation (Hamburg, Germany), STAT-1 and phospho-STAT-1 (Tyr701) were purchased from Cell Signaling (Boston, MA), PE-labeled anti-IFNAR2 from PBL Assay Science (Piscataway, NJ, USA), anti-MHC-I (clone W/32) and anti-HLA-DR (clone L243) were kindly provided by S. Stevanović (Tübingen, Germany) [18], Antibodies to NKG2D ligands (NKG2DL) were provided by A. Steinle (Frankfurt, Germany),

BV421 anti-human PD-L1 (clone MIH1) was obtained from BD Biosciences (Franklin Lakes, NJ), and actin antibody was purchased from Santa Cruz (Dallas, TX). The MxA antibody used for immunoblot analysis in Fig. 1B was provided by J. Pavlovic (Zurich, Switzerland). All other immunoblot analyses were performed with a MxA antibody provided by O. Haller (Freiburg, Germany). The MxA antibody for immunohistochemistry and immunofluorescence was purchased from Thermo Fisher Scientific (Waltham, MA).

Real-time PCR

Total RNA was prepared using the NucleoSpin RNA II System (Macherey-Nagel AG, Düren, Germany) and transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA). Gene expression was measured in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with SYBR Green Master Mix (Thermo Fisher Scientific). The conditions for the PCR reactions were: 40 cycles, 95°C/15 sec, 60°C/1 min, using the following specific primers (Microsynth AG, Balgach, Switzerland): *GAPDH* forward: 5'-CTCTCTGCTCCTCCTGTTCGAC-3' (NM_002046.4, nt 100-121), *GAPDH* reverse: 5'-TGAGCGATGTGGCTCGGC T -3' (NM_002046.4, nt 150-168); *IFNAR1* forward: 5'-TATGCTGCGAAAGTCTTCTTGAG-3' (NM_000629.2, nt 1510-1532), *IFNAR1* reverse: 5'-TCTTGGCTAGTTTGGGAACTGTA-3' (NM_000629.2; nt 1726-1748); *IFNAR2* forward: 5'-TCTTGAGGCAAGGTCTCGCTA-3' (NM_207584.2, nt 1195-1215), *IFNAR2* reverse: 5'-CAGGGATGCACGCTTGTAATC-3' (NM_207584.2, nt 1320-1340); *IFN-α14* forward: 5'-TCTTCGGGATTCCCAATGGC-3' (NM_002172.2; nt 30-49), *IFN-α14* reverse: 5'-CTTGACTTGCAGCTGAGCAC-3' (NM_002172.2; nt 81-100); *IFN-β1* forward: 5'-AGTAGGCGACACTGTTCGTG-3' (NM_002176.3; nt 67-86), *IFN-β1* reverse: 5-AGCCTCCCATTCAATTGCCA-3' (NM_002176.3; nt 221-240); *MxA* forward: 5`-TGGAGATCAGCTCCCGAGATG-3' (NM_001144925.1, nt 512-532), *MxA* reverse: 5'-

ATTGCCCACAGCCACTCTG-3' (NM_001144925.1, nt 570-588). Data analysis was done using the ΔΔCT method for relative quantification.

RNA interference-mediated gene silencing

For transient transfections, glioma cells were seeded in a six-well plate and transfected with 100 nM of specific or scrambled control small interfering (si) RNA by electroporation using the NeonTM Transfection System (Invitrogen). Control as well as *MxA*, *IFNAR1* and *IFNAR2* siRNA oligonculeotides were purchased from Thermo Scientific using siGENOME SMARTpool. siRNA oligonucleotides targeting *IFN-α14, IFN-α17* or *IFN-β1* were obtained from Qiagen.

Flow cytometry

Cells were detached using accutase (PAA, Vienna, Austria), incubated with primary antibody for 30 min at 4°C followed by an R-phycoeythrin-conjugated secondary antibody labeling step for another 30 min in case of not unlabeled primary antibody. Fluorescence was detected in a Cyan flow cytometer (Beckman Coulter, Nyon, Switzerland) or a BD FACSVerseTM flow cytometer. Signal intensity was calculated as the ratio of the median fluorescence of the specific antibody and the isotype control antibody (specific fluorescence index (SFI)).

Immune cell lysis assay

Immune-mediated glioma cell lysis was determined using a flow cytometry-based cytotoxicity assay [17]. Target cells were stained with PKH-26 (Sigma-Aldrich) for 3 min and then co-incubated with NKL effector cells at different effector to target (E:T) ratios as indicated for 4 h. Subsequently, live/dead staining was performed with Zombie Aqua Fixable Viability Kit (BioLegend, San Diego, CA) followed by assessment of target cell lysis by flow cytometry. Specific cell lysis was expressed as percentage of dead cells within the PKH-26-

positive target cells, corrected for spontaneous background lysis. For PD-L1 blocking experiments, T-325 or ZH-161 cells were incubated 1 h prior and during co-incubation with 10 µg/ml of anti-PD-L1 (clone 29E.2A3, Biolegend).

Immunoblot analysis

The cells were treated as indicated and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate) containing phosphatase inhibitor cocktails 2 and 3 and protease inhibitor (Roche Diagnostics, Grenzach-Wyhlen, Germany). Thirty µg of protein per lane were mixed with Laemmli buffer containing β-mercaptoethanol and analyzed by immunoblot. Visualization of protein bands was accomplished using horseradish peroxidase-coupled secondary antibodies (Santa Cruz) and enhanced chemiluminescence (Thermo Fisher Scientific).

Immunohistochemistry

Immunohistochemistry was carried out on tissue microarrays (TMA) with tissue specimens of normal brain or of gliomas of different World Health Organization (WHO) grades of malignancy that were kindly provided by Karl Frei (Department of Neurosurgery)[19]. TMA sections were stained using the rabbit polyclonal primary antibody to MxA (1:200) and visualized using anti-rabbit secondary antibody with Imm Pact DAB kit (Vector, Burlingame, CA). The intensity of staining and the percentage of stained tumor cells on the TMA were quantified by H scoring [20,21]. For co-stainings, TMA sections were stained with primary antibodies to MxA (rabbit, 1:200) and CD45 (mouse, 1:50) and visualized using mouse anti-rabbit IgG-HRP secondary antibody (Santa Cruz) with ImmPact DAB kit (Vector Laboratories, Burlingame, Ca) followed by anti-mouse IgG-AP secondary antibody (Vector Laboratories) with HighDef greenIHC chromogen kit (Enzo, Lasuen, Switzerland).

Immunofluorescence

Five x 10^5 cells were cytospun onto a glass slide, dried for 30 min, fixed in ice-cold methanol for 10 min and permeabilized with 0.5 % Triton X100. Subsequently, cells were stained using the rabbit polyclonal primary antibody to MxA (1:100), visualized using an AF488-conjugated secondary antibody (Southern Biotech, Birmingham, AL) and mounted in Vectashield Mounting Media with DAPI (Burlingame, CA).

TCGA analysis

Kaplan-Meier analysis of survival probability for the glioblastoma 540 - MAS5.0 - u133a dataset (n=540) within the TCGA database (http://cancergenome.nih.gov) was done using the R2: microarray analysis and visualization platform (http://r2.amc.nl) and median MxA expression was selected as scan cut-off mode. 85 samples of this dataset classified according to glioblastoma subtype [22] were analyzed for MxA expression using the subtype track mode [23]. Correlation analysis for the glioblastoma 540 - MAS5.0 - u133a dataset (n=540) or the Glioma - French - 284 - MAS5.0 - u133p2 dataset (n=284) was done using the module for correlation of 2 genes of the R2: microarray analysis and visualization platform (http://r2.amc.nl). Samples of the Glioma - French - 284 - MAS5.0 - u133p2 dataset (n=284) were grouped according to the different WHO grades of malignancy and analyzed for MxA expression using the histology subtype track mode.

Data analysis

Data are representative of at least two independent experiments. If not indicated otherwise, analysis of significance was performed using the two-tailed Student's t-test (* $p < 0.05$; ** $p < 0.01$).

Results

STAT1 phosphorylation and MxA expression indicate autocrine IFN signaling in glioma cells

Binding of type I IFN to their cognate cell surface receptor induces down-stream signaling through STAT1/STAT2 and Jak1/Tyk2 kinases. To demonstrate constitutive IFN signaling as well as responsiveness to exogenous IFN, we assessed expression of MxA, an established marker for activation of the classical IFN-α/β signaling pathway. Highest constitutive MxA levels were seen in T-325 and ZH-161 GIC cells, and MxA mRNA levels increased in all cell lines upon exposure to IFN-β (Fig. 1A). Several glioma cell lines displayed constitutive MxA protein expression with T-325 and ZH-161 GIC having the highest constitutive MxA protein levels. Exposure to IFN-β induced MxA expression in all cell lines investigated (Fig. 1B,C). Furthermore, we determined the basal levels of phosphorylated STAT1 as well as the induction of STAT1 phosphorylation upon exposure to recombinant IFN-β. We observed constitutive STAT1 phosphorylation to a variable degree in all cell lines and an induction of STAT1 phosphorylation upon exposure to exogenous IFN-β (Fig 1B).

To rule out an artificial effect of the cell culture medium in the induction of IFN-β-target genes such as MxA, we cultured LTC in NB medium: here, no induction of MxA was observed; further, culturing GIC in serum-free (SF) or complete DMEM medium had no major consistent effect on MxA levels (Fig. 1D), but interfered with the sphere culture phenotype at longer incubation times (data not shown).

The IFN-β signaling pathway is activated in glioma cells in vivo

We then explored whether IFN signaling is constitutively active in gliomas *in vivo*. To this end, we analyzed the expression of the IFN-specific target gene, *MxA*, using data from glioma patients of different WHO grades deposited in the TCGA database. There was a trend for increased MxA mRNA levels with increasing WHO grade with highest levels in glioblastoma

(Fig. 2A, left). No differential expression was observed when MxA levels were examined in the molecular subtypes classified by Verhaak et al. [22] (Suppl. Fig. 1A). The survival of glioblastoma patients with tumors expressing high MxA levels was shorter when the median was used as cut-off (Fig. 2A, right). We observed a strong correlation of MxA expression with the IFN response genes STAT1, STAT2, STAT3 and IFN regulatory factor 7 (IRF7), but not with IFNAR1, IFNAR2 or type I IFN on the transcriptional level (Suppl. Fig. 1B). To confirm the results obtained by the TCGA database interrogation, we analyzed MxA protein levels by immunohistochemistry on a TMA of gliomas of different WHO grades. MxA was mainly found in the cytoplasm. As shown in Fig. 2B and C, MxA protein levels increased with WHO grade. Again, highest MxA levels were observed in glioblastoma. Similarly, pSTAT1 significantly correlated with MxA protein levels (Fig. 2B, right). Since IFN are also produced by activated immune cells, we looked for the presence of MxA-expressing immune cells in the glioma tissue specimens on the TMA, too. Despite the overall low numbers, we noticed an increase of CD45+MxA+ immune cells with histological grade (Fig. 2D).

IFN receptors are expressed by glioma cells

IFN-α and IFN-β mediate their effects through the type I IFN heterodimer cell surface receptor complex composed of two subunits, IFNAR1 and IFNAR2. First, we confirmed that IFNAR1 and IFNAR2 are expressed on a transcriptional level in the panel of glioma cells tested (Fig. 3A, left). Moreover, as observed previously, only IFNAR2, but not IFNAR1, was detectable by flow cytometry using currently available antibodies (Fig. 3A, right) [24].

To confirm that constitutive MxA expression in GIC depends on signaling through IFNAR, we silenced both receptor subunits using RNA interference in T-325 and ZH-161 cells. The knock-down efficacy was confirmed at the mRNA level for both receptor subunits (Fig. 3B, left) and additionally at the protein level for IFNAR2 (Fig. 3B, right).

Silencing of *IFNAR1* or *IFNAR2* resulted in reduced MxA mRNA levels (Fig. 3B, left). In line with these findings, STAT1 phosphorylation and MxA protein levels were reduced upon silencing of *IFNAR1* or *IFNAR2* in both cell lines (Fig. 3C).

Identification of ligands to the type I IFN receptor in glioma cells

Based on the results obtained with IFN receptor silencing, it seemed most likely that endogenous IFN propagates the signaling through IFNAR. To identify the ligands, we determined the expression of IFN-α and IFN-β at mRNA level in glioma cell lines. Both ligands were expressed at variable levels in the panel of glioma cells tested (Fig. 4A). Silencing of *IFN-α* or *IFN-β* by RNA interference resulted in decreased MxA mRNA expression (Fig. 4B), whereas on the protein level, silencing of *IFN-α* or *IFN-β* led to reduced MxA and pSTAT1 protein levels, more prominently by silencing of *IFN-β* than *IFN-α*, confirming an autocrine IFN signaling loop in glioma cells (Fig. 4C).

Autocrine IFN signaling impairs the immunogenicity of glioma cells

Finally, we defined the role of constitutive IFN signaling in human GIC on a functional level focusing on immunological aspects. Disruption of autocrine IFN signaling using siRNA-mediated silencing of *IFNAR1* and *IFNAR2* in T-325 or ZH-161 cells resulted in decreased programmed death-ligand 1 (PD-L1) cell surface expression, whereas addition of exogenous recombinant IFN-β increased PD-L1 cell surface levels (Fig. 5A). Accordingly, using a TCGA database interrogation, we observed a significant correlation between MxA and PD-L1 mRNA expression levels *in vivo* in glioma patients (Fig. 5B). Moreover, MxA levels correlated with the T cell activation marker CD69, but not with CD25 or PD-1 (Suppl. Fig. 2A). In order to define whether MxA is a regulator of PD-L1 expression, we silenced MxA using RNA interference which, however, did not affect PD-L1 cell surface levels (Suppl. Fig. 2B). Silencing of IFNAR1 and IFNAR2 by RNA interference did not influence NKG2DL

levels but resulted in decreased MHC class I and class II cell cell surface expression (Suppl. Fig. 3A, Fig. 5C). In line with these data, we found a significant correlation between MxA and MHC class I and II mRNA expression *in vivo* when data from glioblastoma patients deposited in the TCGA database were analyzed (Suppl. Fig. 3B). MxA mRNA levels also correlated with the expression of the non-classical MHC molecules HLA-E and HLA-G which have been described as mediators of glioma immune escape (Suppl. Fig. 3C) [25,26]. Most importantly, ZH-161 glioma cells were more susceptible to NKL cell-mediated immune cell lysis upon IFNAR gene silencing corroborating the assumption that constitutive IFN signaling acts as a negative regulator of anti-tumor immune responses (Fig. 5D). Inhibition of the PD-1 pathway using blocking PD-L1 antibodies did not alter NKL cell-mediated lysis of glioma cells (Suppl. Fig. 3D).

Discussion

IFN-β has been used as a therapeutic agent against glioblastoma in small clinical trials which were largely interpreted as promising [27]. However, the molecular mechanisms responsible for potential IFN-mediated effects on glioma cells *in vivo* have remained uncertain and the effect of IFN-β on the more resistant GIC population is subject of current research. Importantly, it has been demonstrated that the efficacy of type I IFN as anti-tumor drugs is affected by constitutive IFN signaling in that the expression and activation of the IFN-stimulated gene factor 3a (ISGF3a) correlated with cellular IFN responsiveness [15,16]. Moreover, chronic IFN signaling induces the proliferation of dormant hematopoietic stem cells, which might also be relevant for cancer stem cells and their impact on sustained tumor growth [16]. Based on these considerations, we investigated the role of autocrine IFN-β signaling in glioma cells including GIC.

We observed constitutive STAT1 phosphorylation in most glioma cell lines and increased phosphorylation after exposure to exogenous IFN-β (Fig. 1B). Constitutively activated STAT proteins have been attributed a role in the malignant phenotype of many tumors including glioblastoma [28] and constitutively activated STATs have been implicated in radioresistance [29,30]. While STATs can be activated by IFN but also other cytokines or growth factors, MxA may be an ideal candidate to specifically assess the activity of the IFN signaling pathway [12,28]. Although single studies suggested a potential role of α-defensin [31] and interleukins [32] in the induction of MxA, it is widely accepted that it is specifically induced by IFN [33]. Thus, as a suitable readout for the presence of IFN activity, we examined the levels of MxA protein in glioma cells. Constitutive MxA expression was mainly observed in GIC lines (Fig. 1B). These results correlated with qPCR results, which demonstrated higher MxA mRNA expression levels in T-325 and ZH-161 GIC lines than in other cell lines (Fig. 1A). Since LTC and GIC lines are cultured under different conditions, we asked whether the cell culture medium would

influence constitutive MxA expression. Culturing LTC in stem cell medium neither induced nor repressed MxA levels in any cell line while FCS-containing complete medium increased (T-325) or decreased (ZH-161) MxA expression in GIC lines (Fig. 1D). Importantly, GIC change their phenotype in FCS-containing medium and become adherent, thus, the effect of this medium on MxA expression might be a consequence of induced cell differentiation rather than a direct response to medium exchange. Based on these findings, and taking into account that not all GIC lines express MxA, we concluded that MxA expression is not an effect of cell culture medium conditions.

We then analyzed MxA levels *in vivo* and observed higher expression in gliomas than in normal tissue, with highest levels in glioblastoma, suggesting that IFN signaling is constitutively active also in gliomas *in vivo* (Fig. 2). No differential expression between the different subtypes classified by Verhaak [22] was observed (Suppl. Fig. 1) suggesting that this is a rather general pathway. Survival analyses of glioblastoma patients from the TCGA database revealed inferior survival for patients with tumors with increased MxA expression, suggesting an impact of constitutively active IFN signaling on the malignant phenotype of these tumors. Similarly, overexpression of IFN/STAT1-related genes may be predictive for poor survival in the proneural subtype of glioblastoma [28,34].

We then confirmed that IFNAR1 and IFNAR2 are expressed in all cell lines and subsequently performed siRNA-mediated gene silencing of *IFNAR1*, *IFNAR2* or both subunits in T-325 and ZH-161 cells. Receptor silencing led to reduced expression of MxA at mRNA and protein level (Fig. 3B, C). Moreover, gene silencing of the ligands, *IFN-α* and *IFN-β*, resulted in decreased MxA expression, too, confirming an autocrine signaling loop in which type I IFN mediate the induction of MxA through IFNAR1/2 (Fig. 4B, C). There was no correlation of IFN receptor or IFN-β expression with MxA levels in glioblastomas in the TCGA database *in vivo*. Hence, a variable activation status of the receptor subunits and/or their ligands and the involvement of other, yet undescribed signaling pathways, might explain this observation.

Further limitations of TCGA analyses include the inability to determine whether mRNA species stem from tumor cells or infiltrating host cells which is particularly relevant when exploring immunological aspects of the disease. On a TMA, we observed generally low, but increasing numbers of tumor-infiltrating lymphocytes with the WHO grade, suggesting that IFN signaling in glioma cells might be induced in an autocrine and paracrine manner (Fig. 2D).

Although typically expressed only at low levels, IFN may exert strong effects on various tissues [16]. Beside their role in the protection of cells from viral infections, constitutive IFN signaling seems to be involved in various cellular responses to other cytokines and growth factors as well as the proper regulation of immune responses [35-37]. Therefore, we aimed at defining the functional impact of the observed constitutive IFN signaling on the immunogenicity of glioma cells. Here, we noticed reduced PD-L1 as well as MHC class I and II expression upon IFNAR1 or IFNAR2 silencing in GIC, suggesting that IFN signaling in these cells may be involved in the interaction between glioma cells and the microenvironment (Fig. 5). Additionally, MxA mRNA levels correlated with MHC class I and II molecules and with the non-classical MHC molecules HLA-E and HLA-G *in vivo* (Suppl. Fig. 3B, C). PD-L1 is expressed by glioma cells *in vitro* and *in vivo* and confers immunosuppressive effects by promoting T cell apoptosis and induction of regulatory T cells [38]. Similarly, high MHC class I and II expression can inhibit innate immune responses against tumor cells [39]. In line with our findings, a role for endogenous IFN-β in the regulation of PD-L1 expression has been described in neurons [40]. The observation that RNA interference-mediated silencing of MxA did not alter PD-L1 expression indicates that PD-L1 expression is regulated by the IFN pathway of MxA (Suppl. Fig. 2B). Moreover, IFN strongly activate monocytes/macrophages and upregulate MHC class I and II expression [41,42]. Furthermore, the expression of the non-classical MHC molecules HLA-E and HLA-G has been linked to immune-inhibitory effects in gliomas. Here, HLA-E mainly inhibits NK cell-mediated lysis while HLA-G interferes with

CD4 and CD8 T cell activity [25,26]. Accordingly, abrogation of IFN signaling in glioma cells by *IFNAR1* and *IFNAR2* gene silencing made these cells more susceptible to immune cell killing (Fig. 5). Since inhibition of the PD-1 pathway using blocking PD-L1 antibodies did not alter NKL cell-mediated lysis of glioma cells (Suppl. Fig. D), it is tempting to speculate that the increased susceptibility to NKL cell-mediated lysis upon IFNAR gene silencing results from decreased MHC class I and class II expression. Hence, our findings suggest that the upregulation of MHC molecules due to constitutive IFN signaling may impair anti-tumor immune responses by NK cells.

Interruption of endogenous IFN signaling did not result in increased NKG2DL expression, which is line with previous findings indicating that exposure to exogenous IFN-β had no effect or decreased NKG2DL cell surface levels [43] (Suppl. Fig. 3A). Thus, whether the boost of immune responses, induced by the treatment with recombinant exogenous IFN-β, results in a net-immune stimulating or net-inhibitory effect might be cell line and context dependent.

In summary, we describe the presence of constitutive IFN signaling in glioma cells which may have an important role in the interaction of these cells with the microenvironment. A more detailed understanding of this intrinsic IFN activity, particularly in the stem cell compartment of gliomas, may lay a basis for the therapeutic targeting of this pathway in order to interfere with the continuous growth of these tumors.

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Figure legends

Figure 1: **IFN-β target genes are expressed in glioma cells.** A. The human LTC LN-18, LN-428, D247MG, LN-319, A172, U87MG, T98G, LN-308 and LN-229, and the GIC T-325, T-269, ZH-161, ZH-305 or S-24 were cultured without or with IFN-β (150 IU/ml) for 48 h and MxA mRNA expression levels were determined using real-time PCR (median expression levels ± SE are shown from 3 independent experiments). B. The cells were treated as in (A). Whole cell lysates were subsequently analyzed for pSTAT1, STAT1 and MxA protein levels by immunoblot using actin as a loading control (1 out of 2 independent experiments is shown). C. LN-308 or ZH-161 cells, untreated or exposed to IFN-β (150 IU/ml) for 48 h, were analyzed for MxA protein levels by immunofluorescence (scale bar, 50 µm). D. LN-308, LN-229, T-325 or ZH-161 cells were cultured in various medium conditions as indicated for 48 h (SF, serum-free DMEM medium; NB, Neurobasal medium). Whole cell lysates were assessed for MxA expression levels using immunoblot. Actin was used as loading control.

Figure 2: MxA is expressed in gliomas *in vivo*. A. MxA mRNA expression levels in gliomas of different WHO grades were analyzed using data from the TCGA database (left). Overall survival analysis within the TCGA database for glioblastoma patients with high versus low MxA expression was performed by Kaplan-Meier analysis. The median was used as cut-off (right). B. MxA protein levels were assessed by immunohistochemistry on a glioma tissue microarray (TMA) and quantified by H scoring (left). pSTAT1 protein levels were analyzed by immunohistochemistry on a TMA and quantified by H scoring. A correlation analysis of pSTAT1 H scores with MxA H scores is shown (right). C. Representative images of normal brain and glioblastoma specimens with low, intermediate or high MxA levels are shown (scale bar, 100 µm or 10 µm for 20x or 40x magnification, respectively). D.

MxA/CD45 co-staining was performed on a glioma TMA and the number of double-positive cells was counted.

Figure 3: MxA expression in human GIC depends on signaling through IFNAR1 and IFNAR2. A. Basal expression levels of IFNAR1 and IFNAR2 were assessed in LN-18, LN-428, D247MG, LN-319, A172, U87MG, T98G, LN-308 and LN-229, T-325, T-269, ZH-161, ZH-305 or S-24 cells by real-time PCR (left) (median expression levels ± SE are shown from 2 independent experiments). Cell surface IFNAR2 protein was analyzed by flow cytometry (1 out of 2 independent experiments is shown). Isotype control antibody (grey) and specific antibody (black) are shown in the histograms (right). B. siRNA-mediated gene silencing of IFNAR1 (si_IFNAR1si), IFNAR2 (si_IFNAR2si) or IFNAR1 and IFNAR2 in parallel (si_IFNAR1/2) in T-325 or ZH-161 cells was performed by electroporation and confirmed for IFNAR1, IFNAR2 and MxA by real-time PCR 24 h post transfection (median expression levels ± SE are shown from 3 independent experiments) and for IFNAR2 by flow cytometry at 48 h following transfection. Isotype control antibody (grey) and specific antibody (black) are shown in the histograms. C. pSTAT1, STAT1 and MxA levels of control, si_IFNAR1, si_IFNAR2 or double knock-down cells (si_IFNAR1/2) were determined 48 h after transfection by immunoblot (1 out of 3 independent experiments is shown).

Figure 4: Glioma-derived IFN-α or IFN-β induces autocrine signaling. A. IFN-α or IFN-β mRNA expression levels were determined in LN-308, LN-229, T-325, T-269, ZH-161, ZH-305 or S-24 cells using real-time PCR (median expression levels ± SE are shown from 2 independent experiments). B, C. siRNA-mediated gene silencing of IFN-α (si_IFN-α) or IFN-β (si_IFN-β) was performed using electroporation in T-325 or ZH-161 cells. MxA mRNA expression was determined 24 h post transfection by real-time PCR (median expression levels ± SE are shown from 2 independent experiments) (B), while pSTAT1, STAT1 and MxA

protein levels were assessed at 48 h following transfection by immunoblot. Actin was used as a loading control (1 out of 3 independent experiments is shown) (C).

Figure 5: Constitutive IFN signaling impairs the immunogenicity of glioma cells. A. siRNA-mediated silencing of IFNAR1/2 or transfection with control oligonucleotides was performed in T-325 or ZH-161 using electroporation (top), or the cells were exposed to 150 IU/ml IFN-β or not (bottom). Cell surface PD-L1 protein levels were assessed after 72 h by flow cytometry (1 out of 3 independent experiments is shown). Isotype control antibody (grey) and specific antibody (black) are shown in the histograms. B. Correlation analysis of MxA with PD-L1 mRNA expression was performed for glioma patients within the TCGA database. Two-tailed Pearson test coefficients (r) and significances (p) are indicated. C, D. siRNA-mediated gene silencing of IFNAR1/2 in T-325 or ZH-161 cells was performed by electroporation. MHC class I or II cell surface protein levels were determined 48 h post transfection by flow cytometry (median expression levels ± SD are shown from 8 independent experiments) (C). ZH-161 cells were used as target cells in a 4 h NKL cell lysis assays at various effector : target (E:T) ratios as indicated 72 h after transfection. The percentage of target cell lysis corrected for spontaneous background lysis is shown (left) (1 out of 4 independent experiments is shown). The knock-down efficiency for IFNAR1 and IFNAR2 gene silencing was confirmed by real-time PCR (right) (D).